Lack of Association Between Common Endothelial Nitric Oxide Synthase Gene Haplotypes and Left Ventricular Hypertrophy in Hypertension

Georgios D. Kitsios,^{1,2} Ioannis Stefanidis,³ and Elias Zintzaras^{1,2}

The endothelial nitric oxide synthase (*NOS3*) gene has been implicated in the pathogenesis of hypertension-related left ventricular hypertrophy (LVH). Candidate-gene studies have examined the role of *NOS3* variation, but reported results are inconsistent. In this study, we investigated the association of three clinically relevant polymorphisms (promoter T786C, intronic 4a/b, and nonsynonymous G894T) in a case-control sample of 230 ethnically homogeneous (Caucasians) patients with essential hypertension, with ($n = 64$) and without ($n = 166$) clinically diagnosed LVH. Haplotype analysis was also performed. In single-marker analyses, no significant associations with LVH were detected by univariate and multivariate regression models. In the haplotype-based association analysis, no common haplotype was associated with the development of LVH. A rare haplotype consisting of the three mutant alleles (C-a-T*) was found to be present only in patients with LVH (3.4%) and not in control hypertensive patients. Despite the biological rationale for the involvement of the *NOS3* gene in LVH, no evidence for a major role of common *NOS3* haplotypic variation was found. Considering the totality of available evidence, single-gene analyses of the *NOS3* gene have not uncovered detectable genetic effects, and pathway-based analyses that examine interactions of multiple loci may be more informative about the complex genetic etiology of LVH.

Dear Editor:

We read with great interest the genetic association study by Vasconcellos *et al.* (2010) that failed to show any association between endothelial nitric oxide synthase (*NOS3*) gene variation and left ventricular hypertrophy (LVH). The biological candidacy of the *NOS3* gene for the development of LVH has been based on a multitude of experimental and epidemiological data: the enzyme's product (NO) can stimulate angiogenesis, reduce cardiomyocyte hypertrophy, and limit the production of extracellular matrix proteins (Murohara *et al.*, 1998; Kim *et al.*, 1999); cumulative evidence has shown that the *NOS3* variants with a major influence on NO formation have been associated with hypertension risk (Zintzaras *et al.*, 2006a; Pereira *et al.*, 2007); and, finally, genomic convergence data (Kitsios and Zintzaras, 2009b) have highlighted the 7q locus harboring the *NOS3* gene as a region with strong linkage signals for left ventricular wall thickness (Tang *et al.*, 2009).

On the basis of similar hypotheses with Vasconcellos *et al.* (2010), we also tried to examine for association between

NOS3 variation (three common variants [T786C-rs2070744, intron 4a/b, and G894T-rs1799983] and their respective haplotypes) and the risk of developing LVH, in a cohort of hypertensive patients derived from a homogeneous population of Caucasian origin (Greeks).

A total of 230 nondiabetic patients with essential hypertension were recruited from the University Hospital of Larissa according to the following criteria: (1) patient age more than 20 years, (2) onset of hypertension before 60 years of age, (3) established hypertension defined either as long-term treatment of the disease or in those previously untreated with systolic blood pressure more than 140 mmHg or diastolic blood pressure more than 90 mmHg, (4) absence of secondary forms of hypertension (after clinical and laboratory work up), and (5) absence of diabetes mellitus or renal failure. Each patient's medical history was obtained using a standardized questionnaire, and a complete physical examination was performed, including measurement of supine systolic blood pressure and diastolic blood pressure and electrocardiogram recording. The diagnosis of LVH was based on Sokolow and Lyon criteria (Sokolow and Lyon,

¹Tufts Medical Center, Institute for Clinical Research and Health Policy Studies, Tufts University School of Medicine, Boston, Massachusetts.

Departments of ²Biomathematics and ³Nephrology, University of Thessaly School of Medicine, Larissa, Greece.

TABLE 1. GENOTYPIC AND HAPLOTYPIC DISTRIBUTIONS OF NITRIC OXIDE SYNTHASE POLYMORPHISMS FOR PATIENTS WITH AND WITHOUT LEFT VENTRICULAR HYPERTROPHY

Polymorphism	Patients with LVH, n (%)	Patients without LVH, n (%)	p	Unadjusted OR (95% CI)	Adjusted OR ^a (95% CI)
T786C					
TT	24 (37)	47 (28)	0.195		
TC	27 (42)	90 (55)			
CC	13 (20)	25 (15)			
786C*carriership				0.68 (0.37–1.25)	0.46 (0.20–1.08)
786C* alleles				0.93 (0.61–1.41)	N/A
Intron 4a/b					
4b/b	41 (64)	120 (73)	0.069		
4a/b	19 (29)	41 (25)			
4a/a	3 (4)	1 (1)			
4a* carriership				1.53 (0.82–2.87)	1.29 (0.57–2.89)
4a* alleles				1.62 (0.94–2.78)	N/A
G894T					
GG	27 (42)	74 (45)	0.464		
GT	24 (37)	67 (41)			
TT	13 (20)	22 (13)			
894T* carriership				1.28 (0.71–2.31)	1.11 (0.50–2.47)
894T* alleles				1.24 (0.81–1.89)	N/A
Haplotypes					
T-b-G*	(42.5)	(48.8)	0.35		
C-b-T*	(24.7)	(27.2)	0.69		
C-a-G*	(10.9)	(12.1)	0.79		
T-b-T*	(10.2)	(6.5)	0.26		
C-b-G*	(2.8)	(4)	0.64		
T-a-G*	(4.2)	(1.1)	0.07		
C-a-T*	(3.4)	(0)	0.008		
Other	(1.2)	(0.5)	–		

Unadjusted and adjusted odds ratios (OR) with the corresponding 95% confidence intervals (CI) for the association of NOS3 genotypes and left ventricular hypertrophy (LVH) are shown: comparisons for the mutant allele carriership and the mutant alleles (786C*, 4a*, and 894T*, respectively).

^aAdjusted ORs by smoking status, pulse pressure, and duration of hypertension.

NOS3, nitric oxide synthase; N/A, nonapplicable.

1949) and echocardiographic findings, confirmed by a clinical cardiologist. A blood sample for biochemical measurements and DNA extraction was taken from each individual. All participants provided written informed consent. Genotyping of each polymorphism was performed by amplification from 50 to 100 ng of genomic DNA. The primer sequences used and the laboratory conditions for genotyping (polymerase chain reaction, restriction enzymes, and agarose electrophoresis) for each NOS3 polymorphism have been previously described (Kim *et al.*, 2003; Awata *et al.*, 2004). Genotyping was performed by laboratory personnel blinded to clinical status.

All statistical analyses were performed using SAS software (v9.1 SAS Institute Inc). The χ^2 -test with one degree of freedom was used to test whether the frequency distribution of genotypes was in Hardy–Weinberg equilibrium ($p \geq 0.05$). Difference in genotype distribution between hypertensive patients with and without LVH was tested using a Fisher's exact test. For each polymorphism, the associations between (1) the mutant alleles (786C*, 4a*, and 894T*, respectively) and (2) the mutant allele carriers, with the presence of LVH, were examined. The associations were indicated as an odds ratio with the corresponding 95% confidence interval. A multivariate logistic regression model was built after back-

ward selection of variables with the potential to confound the association (Lieb *et al.*, 2009). The final model included pulse pressure, smoking status, duration of disease, and the genotype of interest as predictors of LVH. Haplotype analysis was performed using the SHEsis software platform (Shi and He, 2005).

Our study sample included 230 (125 men) hypertensive patients (mean age = 63.9 ± 9.2 years; mean body-mass index = 28.8 ± 4.2 ; median duration of hypertension = 9 years) of which 208 (90.3%) were on antihypertensive medication (diuretics being the most commonly used drug). Sixty-four had LVH (cases) and the remaining 166 patients were free of LVH (control subjects). Pulse pressure and the number of smokers were significantly higher in the case population. The genotype distributions are shown in Table 1 and they were in Hardy–Weinberg equilibrium for all polymorphisms in the control group. No significant associations were detected in univariate or multivariate analyses for each comparison examined. The distribution of the estimated haplotype frequencies for cases and control subjects is also presented in Table 1. Seven major haplotypes with frequencies $>3\%$ were identified. A significant association with LVH was found only for the C-a-T* haplotype ($p = 0.008$), which was present only in patients with LVH (3.4%) and not in control subjects. None

of the remaining and more common haplotypes of the gene was associated with LVH.

Our study replicates the lack of association between common genetic variants and haplotypes in the *NOS3* gene with LVH in patients with essential hypertension, as supported by the Vasconcellos *et al.* (2010) findings. In our analysis, we examined LVH as a binary outcome (defined by standard clinical criteria) to examine the impact of genetic variants on a clinically relevant entity, but we failed to show any significant associations, in univariate and multivariate analyses and in haplotype-based analysis. The only significant association was found for a rare haplotype (C-a-T*) that was present only in patients with LVH and not in control subjects. This same haplotype was present only in three healthy individuals and in no hypertensive patient in the cohort of Vasconcellos *et al.* (2010). The contradictory results and the rarity of this haplotype (possibly carried by only two individuals in our study) warrant cautious interpretation. This haplotype may tag idiosyncratic, rare variation or could represent a false-positive result. However, even if true, such an association has limited generalizability and clinical utility, since a major role of common *NOS3* haplotypic variation in a common condition such as LVH can be excluded.

Testing of haplotypes overcomes some of the problems encountered with using single polymorphisms in genetic association studies, because the interaction of multiple genetic markers within a haplotype could be a key determinant of disease susceptibility rather than the individual polymorphism (Cardon and Abecasis, 2003; Sandrim and Tanus-Santos, 2007). The haplotype analysis approach is expected to be more powerful than single-marker analysis, because of the ancestral structure incarcerated in the distribution of haplotypes (Cardon and Abecasis, 2003; Zintzaras *et al.*, 2006b). Particularly if the markers used define mutations within functional DNA, then the haplotypes composed of these markers can have more of a biological role. In accordance with previous findings, we have recently shown that *NOS3* haplotypes confer increased risk for hypertension (Kitsios and Zintzaras, 2010); however, when the end-organ damage (LVH) is examined, no association is evident.

Despite the strong biological rationale for considering the *NOS3* as a candidate gene in LVH, recent hypothesis-free evidence from a genome-wide association study on hypertension-related LVH did not detect any significant effects of the common *NOS3*-tagging polymorphisms (Arnett *et al.*, 2009). Although small sample sizes and failure to test for rare variants may hamper the detection of true genetic effects, after considering the concordant evidence available to date (Zhu *et al.*, 2005; Vasconcellos *et al.*, 2010), a major pathogenetic role of common variants of the *NOS3* gene on LVH is not plausible. Single-candidate-gene analyses, such as the ones for the *NOS3* gene, may not be sufficient to track the missing heritability of complex traits, even when true biological association is into play (Kitsios and Zintzaras, 2009a). Hypothesis-free approaches with the potential to uncover previously unsuspected pathophysiology, and pathway-based analyses that consider interactions of multiple loci will be needed to promote understanding of the complex genetic etiology of LVH.

Disclosure Statement

No competing financial interests exist.

Acknowledgments

Scientific support for this project was provided through the Tufts Clinical and Translational Science Institute (Tufts CTSI) under funding from the National Institute of Health/National Center for Research Resources (UL1 RR025752). Points of view or opinions in this paper are those of the authors and do not necessarily represent the official position or policies of the Tufts CTSI. Georgios D. Kitsios is a recipient of a career development award from Tufts Medical Center/Pfizer.

References

- Arnett, D.K., Li, N., Tang, W., Rao, D.C., Devereux, R.B., Claas, S.A., Kraemer, R., and Broeckel, U. (2009). Genome-wide association study identifies single-nucleotide polymorphism in *KCNB1* associated with left ventricular mass in humans: the HyperGEN Study. *BMC Med Genet* **10**, 43.
- Awata, T., Neda, T., Iizuka, H., Kurihara, S., Ohkubo, T., Takata, N., Osaki, M., Watanabe, M., Nakashima, Y., Sawa, T., Inukai, K., Inoue, I., Shibuya, M., Mori, K., Yoneya, S., and Katayama, S. (2004). Endothelial nitric oxide synthase gene is associated with diabetic macular edema in type 2 diabetes. *Diabetes Care* **27**, 2184–2190.
- Cardon, L.R., and Abecasis, G.R. (2003). Using haplotype blocks to map human complex trait loci. *Trends Genet* **19**, 135–140.
- Kim, J.U., Chang, H.K., Lee, S.S., Kim, J.W., Kim, K.T., Lee, S.W., and Chung, W.T. (2003). Endothelial nitric oxide synthase gene polymorphisms in Behcet's disease and rheumatic diseases with vasculitis. *Ann Rheum Dis* **62**, 1083–1087.
- Kim, N.N., Villegas, S., Summerour, S.R., and Villarreal, F.J. (1999). Regulation of cardiac fibroblast extracellular matrix production by bradykinin and nitric oxide. *J Mol Cell Cardiol* **31**, 457–466.
- Kitsios, G.D., and Zintzaras, E. (2009a). Genome-wide association studies: hypothesis-free or "engaged"? *Translational Res* **154**, 161–164.
- Kitsios, G.D., and Zintzaras, E. (2009b). Genomic convergence of genome-wide investigations for complex traits. *Ann Hum Genet* **73**, 514–519.
- Kitsios, G.D., and Zintzaras, E. (2010). A *NOS3* haplotype is protective against hypertension in a Caucasian population. *Int J Hypertens* (In press).
- Lieb, W., Xanthakis, V., Sullivan, L.M., Aragam, J., Pencina, M.J., Larson, M.G., Benjamin, E.J., and Vasan, R.S. (2009). Longitudinal tracking of left ventricular mass over the adult life course: clinical correlates of short- and long-term change in the framingham offspring study. *Circulation* **119**, 3085–3092.
- Murohara, T., Asahara, T., Silver, M., Bauters, C., Masuda, H., Kalka, C., Kearney, M., Chen, D., Symes, J.F., Fishman, M.C., Huang, P.L., and Isner, J.M. (1998). Nitric oxide synthase modulates angiogenesis in response to tissue ischemia. *J Clin Invest* **101**, 2567–2578.
- Pereira, T.V., Rudnicki, M., Cheung, B.M., Baum, L., Yamada, Y., Oliveira, P.S., Pereira, A.C., and Krieger, J.E. (2007). Three endothelial nitric oxide (*NOS3*) gene polymorphisms in hypertensive and normotensive individuals: meta-analysis of 53 studies reveals evidence of publication bias. *J Hypertens* **25**, 1763–1774.

- Sandrim, V.C., and Tanus-Santos, J.E. (2007). Haplotype analysis can provide improved clinical information than single genotype analysis. *Thromb Res* **120**, 779.
- Shi, Y.Y., and He, L. (2005). SHEsis, a powerful software platform for analyses of linkage disequilibrium, haplotype construction, and genetic association at polymorphism loci. *Cell Res* **15**, 97–98.
- Sokolow, M., and Lyon, T.P. (1949). The ventricular complex in left ventricular hypertrophy as obtained by unipolar precordial and limb leads. *Am Heart J* **37**, 161–186.
- Tang, W., Devereux, R.B., Li, N., Oberman, A., Kitzman, D.W., Rao, D.C., Hopkins, P.N., Claas, S.A., and Arnett, D.K. (2009). Identification of a pleiotropic locus on chromosome 7q for a composite left ventricular wall thickness factor and body mass index: the HyperGEN Study. *BMC Med Genet* **10**, 40.
- Vasconcellos, V., Lacchini, R., Jacob-Ferreira, A.L., Sales, M.L., Ferreira-Sae, M.C., Schreiber, R., Nadruz, W., and Tanus-Santos, J.E. (2010). Endothelial nitric oxide synthase haplotypes associated with hypertension do not predispose to cardiac hypertrophy. *DNA Cell Biol* **29**, 171–176.
- Zhu, H., Wang, X., Dong, Y., Treiber, F.A., and Snieder, H. (2005). Influence of the *eNOS* gene on development of blood pressure and left ventricular mass: longitudinal findings in multiethnic youth. *Pharmacogenet Genomics* **15**, 669–675.
- Zintzaras, E., Kitsios, G., and Stefanidis, I. (2006a). Endothelial NO synthase gene polymorphisms and hypertension: a meta-analysis. *Hypertension* **48**, 700–710.
- Zintzaras, E., Kitsios, G., and Stefanidis, I. (2006b). Response to endothelial nitric oxide synthase polymorphisms and susceptibility to hypertension: genotype versus haplotype analysis. *Hypertension* **49**, E2.

Address correspondence to:

Georgios D. Kitsios, Ph.D.

Tufts Medical Center

Institute for Clinical Research and Health Policy Studies

Tufts University School of Medicine

800 Washington St.

Tufts MC #63

Boston, MA 02111

E-mail: gkitsios@tuftsmedicalcenter.org

Received for publication February 24, 2010; accepted February 26, 2010.